PATENT
Docket No. 265.00090101

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant(s):	Pasricha et al.	Group Art Unit:	1632
Serial No.:	09/834,110	Examiner:	J. Woitach
Confirmation No.:	5306		
Filed:	April 12, 2001		

For: TREATMENT OF DISORDERS BY IMPLANTING STEM CELLS AND/OR PROGENY THEREOF INTO GASTROINTESTINAL ORGANS

considered 3/17/03 gw

DECLARATION UNDER 37 C.F.R. § 1.132

Assistant Commissioner for Patents
Washington, DC 20231

Dear Sir:

We, Pankaj Jay Pasricha and Maria-Adelaide Micci, declare and say as follows:

1. We are co-inventors of the subject matter claimed in the above-identified U.S. Patent Application Serial No. 09/834,110, filed April 12, 2001.

2. One of us, Pankaj Jay Pasricha, received a M.B; B.S. from the All-India Institute of Medical Sciences, New Delhi, India in 1982 and is currently Chief, Division of Gastroenterology and Hepatology, and Professor of Medicine, Department of Internal Medicine, Anatomy, Neurosciences and Biomedical Engineering, University of Texas Medical Branch, Galveston, Texas. Dr. Pasricha has completed a Residency in Internal Medicine, All-India Institute of Medical Sciences (1983-85); an Internship and Residency in Internal Medicine, Georgetown University-DC General Hospital, Washington, DC (1985-88); a Fellowship in Pulmonary and Critical Care Medicine, Tufts University-New England Medical Center, Boston, MA (1988-90); and Fellowships in Gastroenterology and Advanced and Therapeutic Endoscopy, Johns Hopkins Hospital, Baltimore, MD

(1990-92). He is board certified in Internal Medicine, Pulmonary Medicine and Gastroenterology. Dr. Pasricha's research activities have included extensive work on the biology of the enteric nervous system and visceral pain, clinical disorders of gastrointestinal motility and endoscopic research. He has more than 200 peer-reviewed publications (peer-reviewed publications, abstracts, manuscripts, book chapters, and reviews) and has given numerous talks at symposia on gastroenterology related topics.

3. One of us, Maria-Adelaide Micci, received a Ph.D. from the University of Rome "La Sapienza," Rome, Italy in 1989, and is currently an Assistant Professor, Department of Internal Medicine, Division of Gastroenterology, University of Texas Medical Branch, Galveston, Texas. Dr. Micci's research activities include work on neuronal stem cell transplantation for the treatment of neurodegenerative disorders of the gastrointestinal tract and work exploring the role of the Na^{+}/Ca^{2+} exchanger in the intracellular Ca^{2+} regulation in the retina. She has more than 10 publications in peer-reviewed journals.

4. We have read and are familiar with the Office Action mailed on August 13, 2002 with respect to the above-identified application and make this Declaration in support of the patentability of the claims of patent application Serial No 09/834,110.

5. To overcome issues related to xenotransplantation, our laboratory has isolated CNS-NSC from the brains of embryonic mice genetically engineered to constitutively express the *Escherichia coli* gene LacZ (*Gtrosa-26* mice). These mice express bacterial β-galactosidase in all cells and can therefore be readily distinguished from the host wild type cells. Staged-pregnant female *Gtrosa-26* mice (B6;129S-Gtrosa26; Jackson Laboratories, Bar Harbor, ME) at embryonic day 15 (E15) were used for the isolation of mouse CNS-NSC. Th brains of embryonic mice were removed and the subventricular

zone (SVZ) tissue dissected from each brain hemisphere. Single cell suspensions were made from this tissue using dispase/trypsin treatment and gentle trituration. The fractions were combined, pelleted and resuspended in Neurobasal medium containing B27, 2mM glutamine and penicillin-streptomycin (NB27). After 2-4 hours, the cells were spun down and media replaced with NB27 plus 20 ng/ml fibroblast growth factor (bFGF) and 20 ng/ml epidermal growth factor (EGF) (Promega, Madison, WI). Under these conditions, embryonic CNS-NSC propagated in culture for several weeks retain their undifferentiated state.

CNS-NSC isolated from E15 *Gtrosa-26* mice were plated onto poly-ornithine-coated glass slides and cultured in the absence of the mitogens EGF and FGF for 7 days. Double-label immunofluorescence staining for neuronal nitric oxide synthase (nNOS) and β-tubulin was performed as well as a Western blot analysis of *Gtrosa-26* mice CNS-NSC total protein extract probed with a specific anti-nNOS antibody. Nitric oxide (NO) production was measured as the average relative fluorescence intensity in the presence of L-arginine (1mM; n = 15 cells) or L-NAME (100μM; n = 10 cells). Ionomycin was added at various time points to increase intracellular calcium (a required cofactor for nNOS). These results are shown in Exhibit A.

CNS-NSC isolated from E15 *Gtrosa-26* mice were transplanted into the pylorus of nNOS -/- mice. One week after transplant, cross sections through the pylorus were stained for β-galactosidase. These results are shown in Exhibit B.

6. Exhibit A shows that *Gtrosa-26* mice CNS-NSC cultured *in vitro* express nNOS and produce NO. Differentiated *Gtrosa-26* mice CNS-NSC cultured for 7 days express nNOS as shown by immunofluorescent double-labeling for nNOS (green) and β-

tubulin(red) (A) and western blot analysis of CNS-NSC total proteins extract probed with a specific anti-nNOS antibody (B). The relative fluorescence intensity reflecting NO production by CNS NSC was measured in the presence of L-arginine (1mM; n = 15 cells) or L-NAME (100μM; n = 10 cells). Ionomycin was added at the time indicated by the arrow to increase intracellular calcium (a required cofactor for nNOS). A significant increase in fluorescence intensity was observed in the presence of L-arginine as compared to L-NAME. Data are from one experiment but are representative of three others (C).

7. Exhibit B shows CNS-NSC from *Gtrosa-26* mice can be tracked after transplantation into the gastric pylorus. Clusters of CNS-NSC derived from *Gtrosa-26* mice 1 week after transplantation into the pylorus of nNOS -/- mice are identified by strong staining for β-galactosidase using DAB reaction (indicated by arrow) (A). No β-galactosidase staining is seen in cross sections of the pylorus of nNOS -/- mouse with sham injection (B). In a whole mount section stained for β-galactosidase immediately after transplant into the wall of the pylorus of nNOS -/- mice (C), CNS-NSC stain green. Ganglionic networks of the myenteric plexus (red, MP), stained for PGP9.5 can be seen in the background. Calibration bar = 10 μm. A cross section through the pylorus (D) shows CNS-NSC (green) stained for β-galactosidase 1 week after transplant in proximity to the circular muscle (CM).

8. We submit that one skilled in the art would conclude from the data in Exhibits A and B that CNS-NSCs can be obtained from embryonic mice and are capable of differentiation into neurons. Murine CNS-NSC express nNOS and produce NO when cultured *in vitro* and survive implantation into the gastric pylorus of the mouse.

9. To determine that substance P is released from rat CNS-NSC, our laboratory has measured intracellular calcium in human intestinal smooth muscle cells in co-culture with embryonic rat central nervous system derived neural stem cells (CNS-NSC) in response to the nicotinic agonist DMPP. Cells were loaded with the calcium-sensitive fluorescent indicator Fura-2 and changes in intracellular calcium were measured in intestinal smooth muscle cells in response to bath application of the nicotinic agonist DMPP (20μM). To demonstrate that DMPP is acting on nicotinic receptor, the DMPP-induced response was blocked by the nicotinic receptor antagonist hexamethonium. To demonstrate that substance P released from CNS-NSC is responsible for the activation of smooth muscle cells, the DMPP-induced response was blocked by the substance P receptor antagonist CP-099994-01. The results are shown in Exhibit C.

10. Exhibit C shows that DMPP induces an increase in intracellular calcium in intestinal smooth muscle cells when in co-culture with CNS-NSC (A). This effect is not observed when intestinal smooth muscle cells are cultured without rat CNS-NSC (B). The DMPP-induced response in intestinal smooth muscle cells in co-culture with rat CNS-NSC was blocked by the nicotinic receptor antagonist hexamethonium (C), demonstrating that DMPP is acting on nicotinic receptor. (D) The DMPP-induced response in intestinal smooth muscle cells in co-culture with rat CNS-NSC was blocked by the substance P receptor antagonist CP-099994-01, demonstrating that substance P released from CNS-NSC is responsible for the activation of smooth muscle cells.

11. We submit that one skilled in the art would conclude from the data in Exhibit C that substance P is released from rat CNS-NSC and that this substance P can activate adjacent intestinal smooth muscle cells.

12. To determine the physiological effects of implanted neural stem cells, our laboratory measured the gastric emptying of liquids in nNOS-deficient mice after the implantation of rat CNS-NSC into the pyloric wall compared to the gastric emptying of liquids in control mice. nNOS -/- mice (Jackson Laboratories, Bar Harbor, ME) demonstrate abnormalities in gastric physiology and the gastric emptying of both solids and liquids is significantly delayed in nNOS -/- mice compared with control wild-type mice. To determine gastric emptying of liquids, mice were briefly sedated with diethyl ether, oral-gastric intubation was accomplished with a thin plastic catheter and 0.3 mL solution of 0.05% phenol red was instilled into the stomach. At time points of 0, 5, 10, 20, and 25 minutes, mice were rapidly sacrificed by cervical dislocation. After ligation of the duodenum and transverse resection of the distal esophagus the entire stomach was excised and homogenized with a polytron homogenizer in 10 mL of 0.1 N NaOH. After centrifugation (140,000 g for 15 minutes), an aliquot of the supernatant (0.5 mL) was added to 0.05 mL of 20% trichloroacetic acid. After centrifugation (2500 g for 20 minutes) the supernatant was added to 0.4 mL of 0.5N NaOH and phenol red was measured by reading the absorbance of the sample at 560 nm in a spectrophotometer. Gastric emptying data is presented as the percent of phenol red emptied from the stomach, which is calculated according to the formula: Liquid Gastric Emptying (%) = (1- Absorbance of Test Sample/Absorbance of Baseline Control) X 100, from at least 4 several animals for each time point. The results are shown in Exhibit D.

13. Exhibit D shows gastric emptying of liquids at 20 minutes after gavage of dye in wild type mice (WILD TYPE), nNOS -/- mice (nNOS -/- CTRL), nNOS -/- mice 12 days after injection of PBS into the pylorus (nNOS -/- SHAM), nNOS -/- mice 12 days after transplantation of rat CNS-NSC into the pylorus (nNOS -/- CNS-NSC). Bars indicate standard errors.

14. We submit that one skilled in the art would conclude from the data in Exhibit D that liquid gastric emptying in nNOS -/- mice is improved after transplantation of rat CNS-NSC into the pyloric wall, showing that CNS-NSC transplantation is effective in correcting a neurotransmitter deficiency and producing a beneficial functional effect.

15. We further declare that statements made herein of my knowledge are true, and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

Date: 12/30/02 Pankaj Jay Pasricha

Date: 12/30/02 Maria-Adelaide Micci